# PROTECTION AGAINST CHEMICAL AGENT-INDUCED, SEIZURE-RELATED NEURONAL CELL DEATH

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## INTRODUCTION

Organophosphorus nerve agents are the most toxic chemical warfare agents that present a threat to the warfighter. Irreversible inhibition of acetylcholinesterase, by nerve agents leads to accumulation of the neurotransmitter acetylcholine (ACh) at cholinergic synapses (Taylor, 1996).  Currently fielded MARK I nerve agent antidote for treatment of nerve agent poisoning protects against life threatening consequences (Dunn and Sidell 1989).  When (ACh) accumulates in excess amounts it can initiate seizure activity.  Soldiers surviving the initial life threatening effects of nerve agent poisoning are likely to develop seizure activity. Seizures perturb other neurotransmitter systems and release the excitatory animo acid (EAA) glutamate. The EAA then assumes control of the seizures and progression to status epilepticus (SE) (McDonough and Shih, 1993) .  Sustained release of glutamate and excessive stimulation of EAA receptors triggers delayed secondary excitotoxic biochemical changes involving a complex cascade of factors such as intracellular calcium overload, lipid peroxidation and free radical development, all of which contribute to neuronal cell death (Olney *et al.,* 1983).

Control of seizure activity is the most critical factor in development of brain damage following nerve agent poisoning. While seizure-related brain damage can be prevented by administration of an anticonvulsant drug, battlefield conditions may preclude prompt administration of the convulsant antidote for nerve agents (CANA).  The currently fielded CANA is diazepam.  Diazepam may not prevent or arrest seizures in all individuals.  At the present time there is no capability for measuring seizure activity on the battlefield. Unconscious battlefield victims may undergo silent seizures without convulsive behavioral manifestations.  Delays or failures to locate casualties are to be expected on a battlefield.  Left untreated, seizures progress to status epilepticus and become more refractory to anticonvulsant therapy.    There is a military need for neuroprotective adjunct drugs capable of preventing development of delayed neuronal cell death when administered one or more hours after   onset of seizures.

We have investigated the effects of a synthetic nonpsychotropic derivative of tetrahydrocannabinol (THC), HU-211 (dexanabinol, 7-hydroxy-$\Delta^6$– tetrahydrocannabinol-1, 1-dimethylheptyl; Pharmos Ltd, Rehovot, Israel) on soman-induced seizure-related brain damage.  HU-211 was reported to have neuroprotectant effects in neurons exposed to excitotoxins in culture (Eshhar *et al.,* 1993).   Binding studies revealed that HU-211 blocks N-methyl-D-aspartate (NMDA) receptors in a stereospecific manner at binding sites that are distinct from MK-801 and TCP binding sites (Feigenbaum *et al.,* 1989).  HU-211 was observed to attenuate cell damage produced by nitric oxide and to be a peroxy radical scavenger protecting neuronal cells in culture from free radical generators (Eshhar *et al.,* 1995).  Mechoulam *et al.,* 1998 proposed that the mechanism of action of HU-211 is blockage of the NMDA-operated calcium channel.  HU-211 has been reported to act as a neuroprotectant in animal models of head injury, optic nerve crush and ischemia.  A single injection of HU-211 conferred a significant increase in neuronal survival after the above insults (Mechoulam *et al.*, 1989).


## METHODS

Male Sprague-Dawley rats (CRL: CD[SD]-BR: Charles River Labs, Wilmington MA), weighing between 250-300 g were anesthetized with sodium pentobarbital (35 mg/kg, i.p.) and positioned in a sterotaxic apparatus.  Three holes were drilled through the skull into which screw electrodes were placed for electrocorticographic ECoG recordings.  Electrodes were connected to a standard small-animal headpiece and secured by dental cement.  One week later, the animals were connected to an ECoG recording system and allowed 30 min to acclimate.  Baseline ECoG activity and behavior were

# Report Documentation Page

*Form Approved*
*OMB No. 0704-0188*

| 1. REPORT DATE | 2. REPORT TYPE | 3. DATES COVERED |
|---|---|---|
| **00 JAN 2002** | **N/A** | **-** |

| 4. TITLE AND SUBTITLE | 5a. CONTRACT NUMBER |
|---|---|
| **Protection Against Chemical Agent-Induced, Seizure-Related Neuronal Cell Death** | 5b. GRANT NUMBER |
| | 5c. PROGRAM ELEMENT NUMBER |

| 6. AUTHOR(S) | 5d. PROJECT NUMBER |
|---|---|
| | 5e. TASK NUMBER |
| | 5f. WORK UNIT NUMBER |

| 7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) | 8. PERFORMING ORGANIZATION REPORT NUMBER |
|---|---|
| **La Salle University Philadelphia, PA 19141-1199; US Army Medical Research Institute of Chemical Defense Aberdeen Proving Ground, MD 21010-5400** | |

| 9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) | 10. SPONSOR/MONITOR'S ACRONYM(S) |
|---|---|
| | 11. SPONSOR/MONITOR'S REPORT NUMBER(S) |

**12. DISTRIBUTION/AVAILABILITY STATEMENT**
**Approved for public release, distribution unlimited**

**13. SUPPLEMENTARY NOTES**
**This article is from ADA409494 Proceedings of the 2001 ECBC Scientific Conference on Chemical and Biological Defense Research, 6-8 March , Marriott's Hunt Valley Inn, Hunt Valley, MD.**

**14. ABSTRACT**

**15. SUBJECT TERMS**

| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON |
|---|---|---|---|---|---|
| a. REPORT **unclassified** | b. ABSTRACT **unclassified** | c. THIS PAGE **unclassified** | **UU** | **6** | |

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Prescribed by ANSI Std Z39-18

monitored for at least 15 min. Following baseline recordings, animals were injected (i.p.) with 125 mg/kg of the oxime HI-6. This was followed 30 min later by injection of 180 µg/kg soman (1.6 $LD_{50}$, s.c.) or sterile saline. Within one min following soman or saline injection, animals were injected (i.m.) with 4-mg/kg atropine methylnitrate (AMN). The quaternary compounds, HI-6 and AMN, were administered to protect against the peripheral effects of soman and to ensure survival. HU-211 was injected (25 mg/kg in Miglyol) at 5 or 40 min following onset of seizures as determined by ECoG recordings. In a small group of animals 20 mg/kg, (i.p.) diazepam was administered 40 min after seizure onset or 20 mg/kd diazepam + 25 mg/kg HU-211 were given at this same time.

Twenty-eight hours after soman administration, rats were given an injection of pentobarbital (100 mg/kg, i.p.) and euthanatized via transcardial perfusion with ice cold 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4. Brains were immediately excised and longitudinally divided into left and right hemispheres. Alternate hemispheres were postfixed by immersion in a second solution of ice-cold 4% paraformaldehyde in 0.1M phosphate buffer for 4-6 hr. The hemispheres were subsequently sucrose-saturated (30% sucrose in 0.1M buffer for 72 hr) and coronally sectioned at 40 µm. Serial sections were collected directly onto polylysine-coated slides for staining or cryoprotected and stored at $-20^{o}$ C pending immunocytochemical staining for microtubule associated protein 2 (MAP2). The remaining hemispheres were paraffin processed, sectioned at 4 µm and stained with hematoxylin and eosin (H&E). Morphometric image analysis of MAP2 stained sections was performed according to the procedure described by Ballough *et al.,* (1995).

Group means for temporal lobe lesion volumes obtained from MAP2-stained sections were compared using t-tests. ECoG data were grouped according to treatment, post-seizure onset delay, frequency band and absolute or relative powers. Group means were compared using one-way analysis of variance (ANOVA), followed by the Student-Newman-Keuls (SNK) multiple range test. Chi-square analysis was used to assess the relative power distributions across frequency bands in the 24 hr ECoG data. Regression analysis was used to extract ECoG correlates of seizure-related brain damage or neuroprotection by comparing all powers and relative powers of all frequencies to lesion volumes at 28 hr following soman injections. Values for $p < .05$ were considered significant.


## RESULTS

All soman-treated rats exhibited sustained seizures and status epilepticus for several hr with or without HU-211 treatment. This determination was based on the presence of ECoG amplitudes greater than four times baseline. Proconvulsive behavioral signs of soman intoxication included repetitive chewing, facial clonus, forepaw clonus, motor stereotypy and wet-dog shakes. Overt motor convulsions were characterized by rhythmic clonic jerks of both head and forepaws, rearing, salivation and Straub tail. Seizure activity was evident in these animals when assessed 24 h post-soman administration. ECoGs from non-soman control rats showed no evidence of seizures and there was no histological evidence of neuropathology in H&E- and MAP2-stained brain sections from these animals.

Histopathological evaluations of H&E-stained brain sections from rats that received soman but not HU-211 revealed severe region-specific brain damage, i.e., some regions exhibited severe lesions, while others were virtually unaffected. This damage was bilaterally symmetrical and characterized by widespread tissue necrosis, neuronal loss, chromatolysis, vacuolization, pyknosis and gliosis. The most severe damage was consistently observed in the piriform cortex, entorhinal cortex, dorsal endopiriform nucleus and the laterodorsal thalamic nucleus. Pronounced damage was seen in perirhinal cortex, amygdaloid complex, hippocampus and midline thalamic nuclei. This pattern of soman-induced seizure-related brain damage is consistent with previous reports (Ballough *et al.,* 1995, 1998: McDonough *et al.,* 1998).

Histopathological damage rating for H&E-stained brain sections are based on the presence of necrotic neurons and/or the absence of a defined neuronal population. In this study, piriform cortical brain damage was significantly ($p = .008$) reduced in the soman + HU-211-treated animals compared to soman controls.
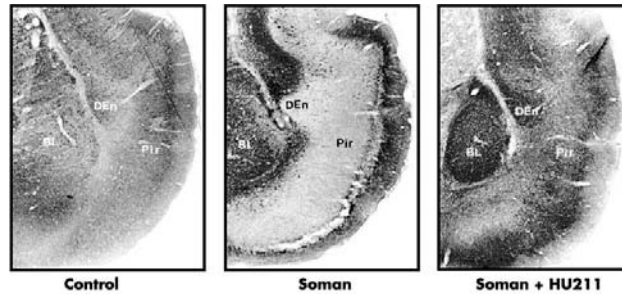
Figure 1. MAP2 immunohistochemical staining of the rat temporal lobe. **<u>Control</u>**: saline-injected animal**; <u>Soman</u>**; soman-induced lesions after 4-5 hr status epilepticus; **<u>Soman + HU-211</u>**: neuroprotection produced by treatment with HU-211. BL: basal lateral amygdala; Den: dorsal endopiriform nucleus; Pir: piriform cortex. Black letters indicate damaged areas.

Figure 1 (Control) shows the MAP2 immunostaining of the rat temporal lobe. In control animals, MAP2 staining was localized in the neuronal perikarya and proximal dendrites. MAP2 immunostaining was not observed in areas composed of white matter, except in small numbers of scattered neurons. The loss of the neuron-specific MAP2 marker indicates severe neuronal damage and provides clear demarcation of widespread neuronal necrosis (Ballough *et al.,* 1995). In this study severely damaged brain regions were easily identified by an almost total absence of MAP2 immunostaining ( Fig 1, soman). Clearly demarcated macroscopic lesions (MAP2-*negative* immunostaining) were consistently observed in the piriform, perirhinal and entorhinal cortices, endopiriform nucleus, postiolateral cortical amygdaloid nucleus, laterodorsal, mediodorsal, ventromedial and lateroposterior thalamic nuclei of the soman control group. Along the anterior-posterior axis of the temporal lobe, cross-sectional areas of necrosis were greatest at bregma $-3.3 \pm 0.2$ mm, irrespective of lesion volume. These lesions often extended dorsally to include the perirhinal cortex. Medially, the area of damage often included the endopiriform nucleus while sparing the lateral and basolateral amygdaloid nuclei. In the most severe cases, the necrotic core included these nuclei as well. In the penumbra, surrounding the necrotic core, MAP2 immunostaining was elevated above controls. Control animals showed no evidence of MAP2 loss in any of the above brain regions. And the MAP2 immunostaining was consistent between individuals and exhibited the normal staining pattern described previously (Ballough *et al.,* 1995, 1998).
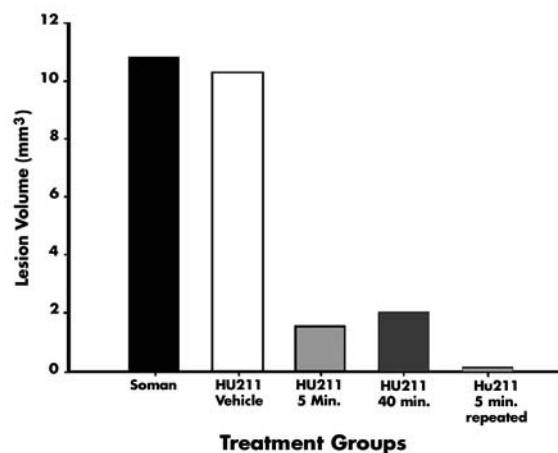


Figure 2. Histogram depicting median lesion volumes of temporal lobe necrosis ($mm^3$). Morphometric image analysis was used to assess MAP2-*negative* staining in the piriform cortex and contiguous regions. HU-211 reduced median lesion volume 86% when administered 5 min after seizure onset; 81.5% at 40 min and repeated administrations (5 min then @ 6 hr) produced a 99% reduction in lesion volume.

HU-211 provided considerable neuroprotection against soman-induced seizure-related brain damage, (Fig 1, soman + HU-211). When administered 5 min after seizure onset, unilateral temporal lobe macroscopic lesion volume was significantly reduced ($p = .006$) in the soman + HU-211 treated group 86% (Fig 2).

When administered 40 min after seizure onset, HU-211 reduced the median lesion volume of necrosis 81.5%. In a small group of animals (n = 6) the HU-211 was given 5 min after seizure onset and then every six hr for the next 24 hr. In this group, median lesion volume was reduced 99% (Fig 2).
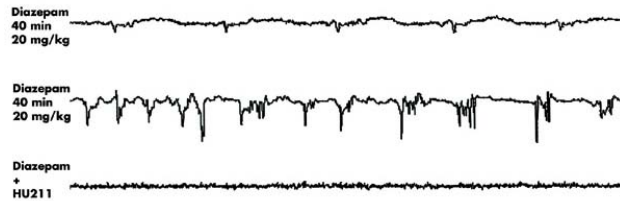
Figure 3. ECoG recordings from animals given 1.6 LD$_{50}$ soman and 20 mg/kg diazepam 40 min after seizure onset. The lower tracing is from an animal that received diazepam at 40 min as above and 25 mg/kg HU-211 immediately after the diazepam.

Figure 3 shows the ECoG records at 24 hr after soman injection of three animals that were given diazepam or diazepam + HU-211 at 40 min after seizure onset. It has been shown by McDonough (personal communication) that diazepam is not effective in arresting seizures when given 40 min after seizure onset even at very high doses, e.g., 20 mg/kg. When HU-211 was administered immediately following the diazepam, seizure activity abated.

Statistical analysis of ECoG total power (i.e., sum of all frequencies) revealed no differences between the soman control and the soman + HU-211 groups at any of the sampling periods. Spectral power analysis revealed that relative Delta-1 power (0-0.5 Hz) in soman controls increased more than three fold over the baseline value of this frequency. In the soman + HU-211 group, the 24 hr relative delta-1 power was not significantly different from the baseline value.

The most striking fluctuation in the ECoG power of all frequency bands was seen in relative beta-2 (21-31.5 Hz). Chi square analysis of the relative power distributions across frequencies revealed a significant shift of the entire ECoG spectrum toward the lower frequencies in the soman control group compared to the HU-211 protected group at the 24 hr time period. Regression analysis provided evidence of a shift in the entire ECoG spectrum: there was a good correlation ($R^2 = 84.4$, $p < .001$) between decreased relative beta and increased relative delta in the soman control group. Of 109 regression analyses, using ECoG powers and relative powers of all frequency bands and total power at all time points as predictors of subsequent lesion volume at 28 hr, only 15 frequency/time points had correlations coefficients above 20. There were 5 frequency/time-points with correlations above 30, and 2 with correlations above 34.5, i.e., relative beta-1 ($R^2 = 58.5$, $p < .01$) and relative beta-2 ($R^2 = 90.6$, $p < .001$) at 60 min after onset of seizures (Fig 4).
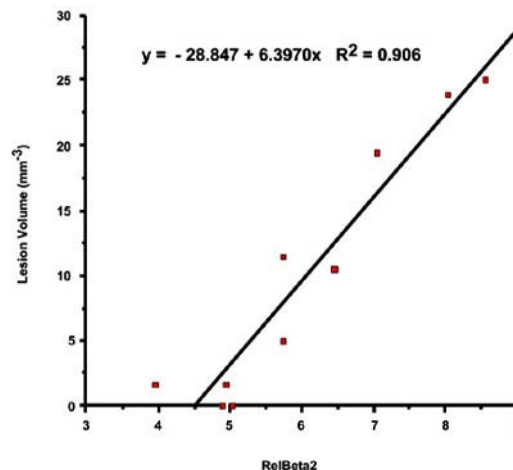


Figure 4. Correlation of median lesion volumes observed at 27 hr after soman injection with relative beta-2 frequency power at 60 min after seizure onset (P < .001).

DISCUSSION

As see in Fig 1, HU-211 conferred considerable protection against brain damage resulting from soman-induced seizures without diminishing the intensity or duration of the seizures. MAP2 volumetric assessments revealed an 86%

reduction in temporal lobe macroscopic lesions in the HU-211-protected group compared to soman controls. This observation was supported by H&E histopathological evaluations where the HU-211-treated animals showed a significant reduction in piriform cortical damage. The HU-211 group showed only a mild neuronal loss (11-25%) compared to soman controls which had severe neuronal loss (> 45%).

Earlier reports have shown that NMDA antagonists such as ketamine and MK-801 protect thalamic neurons from seizure-related brain damage without preventing seizure activity (Clifford et al., 1990). These authors suggested that these NMDA antagonists may have prevented seizure-related damage by blocking NMDA receptor ion channel complexes on the dendrosomal surfaces through which glutamate excitotoxicity is expressed and that the seizure activity can be maintained by other neurotransmitter systems without NMDA receptor participation.

The high correlation of lesion volume with relative power of beta-2 frequency observed at 60 min after seizure onset may indicate that this is the absolute window for preventing seizure-related brain damage. This time point coincides with the irreversible effects of intracellular calcium overload that leads to cell death (Randall and Thayer, 1992).

Interestingly, neither HU-211 nor diazepam administered at 40 min after seizure onset stops the seizure activity when administered alone. But when given together, seizures were arrested suggesting a synergistic interaction between these two compounds. This synergy may have produced augmentated hyperpolarization of post synaptic memebranes by the combined HU-211 antagonnism of NMDA receptors and agonistic modulation of the GABA receptors by diazepam.

The results presented here demonstrate that pharmacological interventions can prevent or minimize delayed brain damage resulting from chemical warfare agent-induced seizure activity. Such intervention is most important where administration of countermeasures for chemical warfare agents is delayed. HU-211 or other neuroprotectant drugs may be useful adjuncts for medical management of chemical casualties on the battlefield.

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